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# rIFN-γ-mediated growth suppression of platinum-sensitive and -resistant ovarian tumor cell lines not dependent upon arginase inhibition

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## Abstract

**Background:** Arginine metabolism in tumor cell lines can be influenced by various cytokines, including recombinant human interferon-γ (rIFN-γ), a cytokine that shows promising clinical activity in epithelial ovarian cancer (EOC).

**Methods:** We examined EOC cell lines for the expression of arginase in an enzymatic assay and for transcripts of arginase I and II, inducible nitric oxide synthase (iNOS), and indoleamine 2,3-dioxygenase (IDO) by reverse transcription-polymerase chain reaction. The effects of rIFN-γ on arginase activity and on tumor cell growth inhibition were determined by measuring [$^3$H]thymidine uptake.

**Results:** Elevated arginase activity was detected in 5 of 8 tumor cell lines, and analysis at the transcriptional level showed that arginase II was involved but arginase I was not. rIFN-γ reduced arginase activity in 3 EOC cell lines but increased activity in the 2008 cell line and its platinum-resistant subline, 2008.C13. iNOS transcripts were not detected in rIFN-γ-treated or untreated cell lines. In contrast, IDO activity was induced or increased by rIFN-γ. Suppression of arginase activity by rIFN-γ in certain cell lines suggested that such inhibition might contribute to its antiproliferative effects. However, supplementation of the medium with polyamine pathway products did not interfere with the growth-inhibitory effects of rIFN-γ EOC cells.

**Conclusions:** Increased arginase activity, specifically identified with arginase II, is present in most of the tested EOC cell lines. rIFN-γ inhibits or stimulates arginase activity in certain EOC cell lines, though the decrease in arginase activity does not appear to be associated with the *in vitro* antiproliferative activity of rIFN-γ. Since cells within the stroma of EOC tissues could also contribute to arginine metabolism following treatment with rIFN-γ or rIFN-γ-inducers, it would be helpful to examine these effects *in vivo*.

## Background

Arginine metabolism to nitric oxide (NO) via nitric oxide synthase or to ornithine via arginase is an important biological pathway (see Fig. 1). NO has many important functions, including regulating vascular tone and facilitating cell-mediated cytotoxicity [1], and ornithine is the immediate precursor of polyamines, which are important in cell proliferation [2]. In addition to competition for the substrate, L-arginine, there are other interactions between the two pathways of arginine metabolism, such as the inhibition of arginase by $N^G$-hydroxy-L-arginine, an intermediate in NO biosynthesis [3,4].

Under different experimental conditions, NO may either inhibit [5,6] or promote [7] tumor growth. On the other hand, polyamines appear to be necessary for the proliferation of neoplastic cells, and polyamine depletion results in tumor growth arrest [8]. Inducible nitric oxide synthase (iNOS) catalyzes the production of NO, which contributes to the antitumor activity of activated macrophages [9]. iNOS expression is induced by cytokines, primarily the pro-inflammatory cytokine rIFN-α [9] which may be produced by certain T-lymphocytes and natural killer (NK) cells that are important in adaptive and innate immune responses, respectively [12]. iNOS and arginase are differentially regulated by pro- and anti-inflammatory cytokines [10,11]. In addition, rIFN-γ(rIFN) can have a direct antiproliferative effect on tumor cell lines *in vitro*, including a number of ovarian cancer cell lines [13–15]. This antiproliferative activity in some experimental systems depends on the catabolism of another amino acid, L-tryptophan, by indoleamine 2,3-dioxygenase (IDO) [13,14,16,17]. The antitumor activity of rIFN-γ, administered either intraperitoneally [18] or systemically [19], has also been observed in clinical trials involving epithelial ovarian carcinoma (EOC).

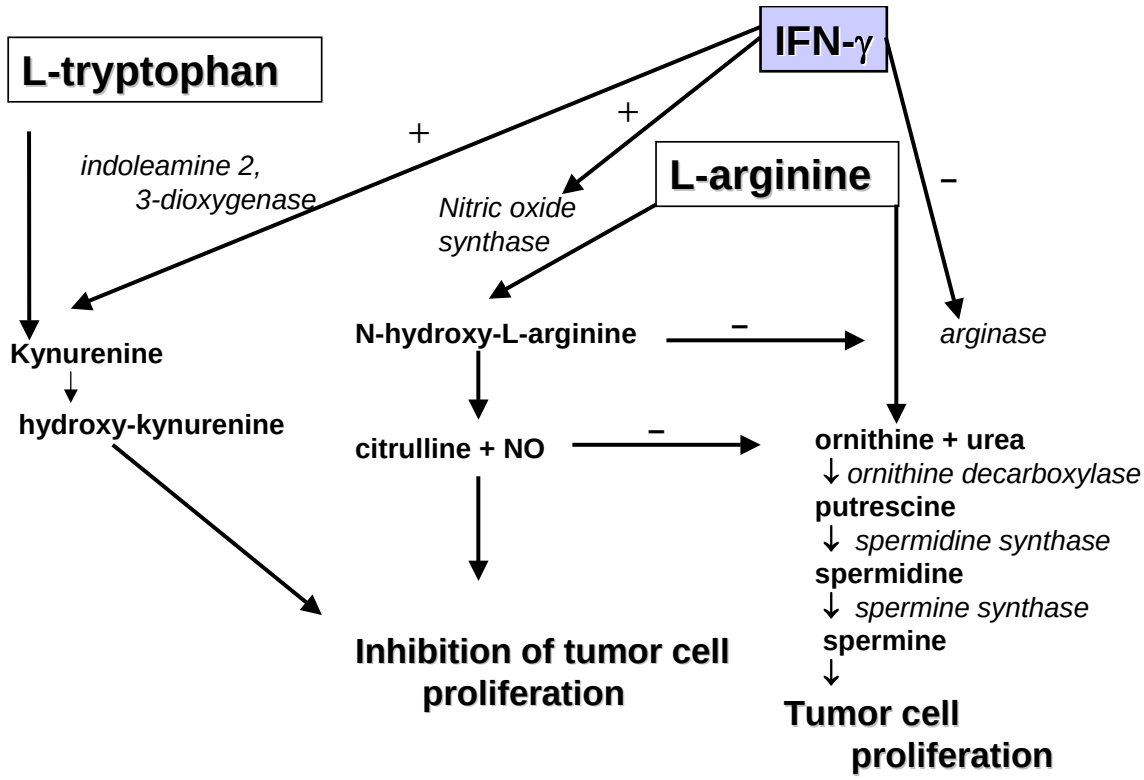


**Figure 1**

**Arginine pathway.** L-arginine is metabolized either by nitric oxide synthase to N-hydroxy-L-arginine and NO, or by arginase to ornithine and urea. IFN-γ induces nitric oxide synthase, but inhibits arginase. N-hydroxy-L-arginine inhibits arginase, and NO inhibits ornithine decarboxylase.

In this study, we examined L-arginine metabolism in EOC cell lines and the effect of rIFN-γ on arginase activity. Arginase expression varied among EOC cell lines. In some cell lines, rIFN-γ reduced arginase activity and expression; however, no direct relationship between arginase activity and the growth inhibitory effects of rIFN-γ was observed.

## Methods

### *Reagents*

rIFN-γ (Actimmune) was obtained from Intermune Pharmaceuticals (Burlingame, CA). L-$N^G$-monomethyl arginine citrate (NMMA) was obtained from Cayman Chemical Company (Ann Arbor, MI) $N^G$-hydroxy-L-arginine was obtained from Alexis (Carlsbad, CA). Ornithine, putrescine, and spermidine were obtained from Sigma (St. Louis, MO).

### *Cell lines*

The 2774 cell line was originally isolated from an ovarian cancer patient in the laboratory of Dr. J. Sinkovics as previously described [20]. The NMP-1 cell line is a moderately platinum-resistant tumor cell line developed from the parental OVCAR3 line by in vitro incubation of the cell with increasing cisplatin concentrations and subsequent passage in the nude mouse in the laboratory of Dr. Jim Klostergaard at The University of Texas M. D. Anderson Cancer Center [21]. The HEY line was originally derived from a human ovarian cancer xenograft, and is characterized by moderate resistance to cisplatin [22]. Both the HEY and NMP-1 cell lines were obtained from Dr. Klostergaard. The 2008 cell line and its platinum-resistant subline, 2008.C13 [23], were obtained from Dr. Zahid Siddik, M. D. Anderson Cancer Center. $CAOV_3$and $SKOV_3$, which overexpress HER2/neu, were obtained from American Type Culture Collection (Manassas, VA). All cell lines were grown in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (Sigma) at 37°C in 5% $CO_2$. 2774 and 2008 are sensitive to platinum. The remaining lines exhibit varying degrees of resistance to platinum.

### *Measurement of arginase activity*

Arginase activity was measured as previously described [24]. Briefly, $5 \times 10^5$ cells were obtained by trypsinization and washed twice with phosphate-buffered saline (PBS, GIBCO). To each sample, 50 ml of 0.1% Triton X-100 (Curtin-Matheson, Houston, TX) containing 5 μg pepstatin, 5 μg aprotinin, and 5 μg of antipain (Sigma) were added, and the samples were shaken for 1 hour at room temperature. An aliquot was then taken for the determination of protein content, 50 μl of 10 mM manganese chloride (Sigma) in 50 mM Tris-HCl (Fisher, Fair Lawn, NJ), pH 7.5, were then added to the sample and the samples were incubated for 10 minutes at 55°C. Afterwards, 25 μl of the sample was transferred to Eppendorf tubes, 25 μl of 0.5 M arginine (Sigma), pH 9.7, was added, and the mixture was incubated at 37°C. After 1 hour, the reaction was stopped by 400 μl of acid mixture (sulfuric acid, phosphoric acid, and water 1:3:7), 25 μl of 9% α-isonitrosopropiophenone in ethanol (Sigma), was added and the samples were heated at 100°C for 1 hour. After 10 minutes in the dark, optic density at 540 nm was determined using a microplate reader. A calibration curve was prepared using increasing concentrations of urea (Curtin-Matheson). In certain experiments, NMMA, an iNOS inhibitor, was added to study a possible role of iNOS induction in the suppression of arginase activity by rIFN-γ.

The protein content of each sample was measured using a bicinchoninic acid method with a commercial kit (Pierce, Rockford, IL) according the manufacturer's instructions. The arginase activity was then expressed as μg of urea per mg of protein.

### *Immunocytochemistry*

The expression of Ki-67, a marker of proliferation, on cultured EOC cell lines was evaluated by immunocytochemistry [25]. Briefly, cytospin preparations of cultured EOC cell lines were fixed with acetone at room temperature and air-dried. After blocking with 0.3% hydrogen peroxide in methanol, the slides were washed twice with PBS and blocked with 1% horse serum. The slides were then incubated with Ki-67 mouse anti-human monoclonal antibody (Pharmingen, San Diego, CA) for 1 hour. The slides were washed twice with PBS and then incubated with a biotin-conjugated secondary antibody for 1 hour at room temperature. After two washes with PBS, the slides were stained using an avidin-biotin-peroxidase complex staining kit (Vector Laboratories, Burlingame, CA) following the manufacturer's instructions. After counterstaining with hematoxylin QS H-3404 (Vector), the proportion of positively stained cells was measured.

### *RNA extraction*

The RNA extraction was performed using TRIzol (Invitrogen Life Technologies, Carlsbad, CA). The procedure was performed according the manufacturer's instructions.

### *Reverse transcription-polymerase chain reaction*

The expression of iNOS, arginase I, and arginase II in ovarian cancer cells treated with and without IFN-γ was examined using a reverse transcription-polymerase chain reaction (RT-PCR).

Reverse transcription was performed using 1–5 μg of total RNA diluted in 11.5 μl of diethylpyrocarbamate-treated water. One microliter of random primers (GIBCO) were added to each RNA sample in a sterile PCR tube. The mixture was then heated at 70°C for 10 minutes. Immediately afterward, the samples were chilled on ice, and 4 μl of

first-strand buffer (GIBCO), 2 μl of 0.1 M DTT (GIBCO), 1 μl of RNse inhibitor (Roche, Mannheim, Germany) and 0.5 μl of 25 mM dNTP (GIBCO) were added to each sample. The samples were then incubated for 10 minutes at 25°C and 2 minutes at 42°C. One microliter of reverse transcriptase (Superscript II, GIBCO) was then added, and the reaction was continued at 42°C for another 45 minutes. The reaction was then stopped by heating the tubes at 95°C for 5 minutes, and the complementary DNA (cDNA) obtained was stored at -20°C until needed for further analysis.

The cDNA samples were then amplified using a polymerase chain reaction [26]. The following primers were used (GIBCO):

iNOS sense 5'-CATGGCTTGCCCCTGGAAGTTTCT-3',

antisense 5'-CCTCTATGGTGCCATCGGGCATC-3';

arginase I sense 5'-CTCTAAGGGACAGCCTCGAGGA-3',

antisense 5'-TGGGTTCACTTCCATGATATCTA-3';

arginase II sense 5'-ATGTCCCTAAGGGGCAGCCTCTCG-CGT-3',

antisense 5'-CACAGCTGTAGCCATCTGACACAGCTC-3';

IDO sense 5'-CCTGACTTATGAGAACATGGACGT-3',

antisense 5'-ATACACCAGACCGTCTGATAGCTG-3';

β-actin sense, 5'-GTTGCTATCCAGGCTGTGC-3',

and antisense, 5'-GCATCCTGTCGGCAATGC-3'.

The amplification was performed in 25 μl of reaction mixture containing primers, 1 μl dimethyl sulfoxide (Sigma), PCR buffer, 0.2 mM dNTP, 1.5 mM magnesium chloride, and 5 U Taq DNA polymerase (GIBCO) under amplification conditions described previously [27,28]. The final PCR product was loaded on a 1.5 % agarose gel, separated by electrophoresis, and visualized using ethidium bromide staining under ultraviolet light. The expected sizes of the amplified fragments were: iNOS, 747 base pairs (bp); arginase I, 794 bp; arginase II, 342 bp; IDO,321 bp; GAPDH, 558 bp; and β-actin, 567 bp. The semi-quantitative measurement of transcript expression was analyzed using a Scion image tool. The relative arbitrary units of transcript expression were normalized using the value of β-actin. GAPDH was used to control the quality of cDNA in certain experiments.

### *Measurement of growth inhibition*

The growth inhibitory activity of rIFN-γ was examined by inhibition of [$^3$H]thymidine incorporation [29]. Briefly, 5000 cells were seeded into flat-bottom 96-well plates (Becton Dickinson, Franklin Lakes, NJ) and incubated with the agent of interest in RPMI 1640 supplemented with 10% fetal calf serum for 72 hours. For the last 16 hours, the cells were pulsed with 50 μl of [$^3$H]thymidine (10 μCi/ml, specific activity 185 Bq/mmol, Amersham, Piscataway, NJ). The cytostasis experiment was then harvested. The cells were washed 3 times with phosphate-buffered saline (GIBCO), and subsequently lysed with 150 μl of 0.1 M sodium hydroxide (Curtin Matheson). The lysate was subsequently transferred to 3 ml scintillation liquid (Safety-Solve, Research Products International, Mount Prospect, IL), and radioactivity was determined using a WinSpectral liquid scintillation counter (EF&G Wallac, Turku, Finland). The percentage of cytostasis for each treatment was calculated using the formula:

$$(cpm_{control\ cells} - cpm_{treated\ cells}) \times 100/cpm_{control\ cells}.$$

In certain experiments, the polyamines (ornithine, putrescine and spermidine) were added to study the effect of polyamines on rIFN-γ-induced cytostasis. $N^G$-hydroxy-L-arginine, an arginase inhibitor, was added to study the effect of arginase suppression on the proliferation of EOC cell lines.

## Results and Discussion

Arginase activity varied among EOC cell lines. There was no apparent relationship between the expression of arginase activity in EOC tumor cell lines and the expression of the Ki-67 antigen. Arginase activity was lower (less than 0.2 μg urea per mg protein) in 3 EOC cell lines – HEY, SKOV3, and OVCAR3 and higher in 2774, $CAOV_3$, NMP-1, and 2008, and in its platinum-resistant subline, 2008.C13 (Table 1). Arginase activity was also higher in the NMP-1 cell line, the cisplatin-resistant subline of OVCAR3. Arginase activity decreased markedly after treatment of 2774, NMP-1, and CAOV3 cells with rIFN-γ. In contrast, rIFN-γ treatment leads to an increase of arginase activity in 2008 and its cisplatin-resistant subline 2008.C13. iNOS expression was examined because $N^G$-hydroxy-L-arginine and NO produced by the activity of this enzyme are known inhibitors of arginase activity. However, the addition of NMMA, an iNOS inhibitor did not abolish rIFN-γ-induced changes in arginase activity (Table 2), which is consistent with the RT-PCR data showing an absence of iNOS expression. Analysis of mRNA transcripts from all 6 EOC cell lines shows that arginase II was the enzyme responsible for the arginase activity (Fig. 2A). Treatment of the cell cultures with rIFN-γ did not increase arginase II transcript expression in any of the 6 cell lines tested. IDO, which is constitutively expressed in

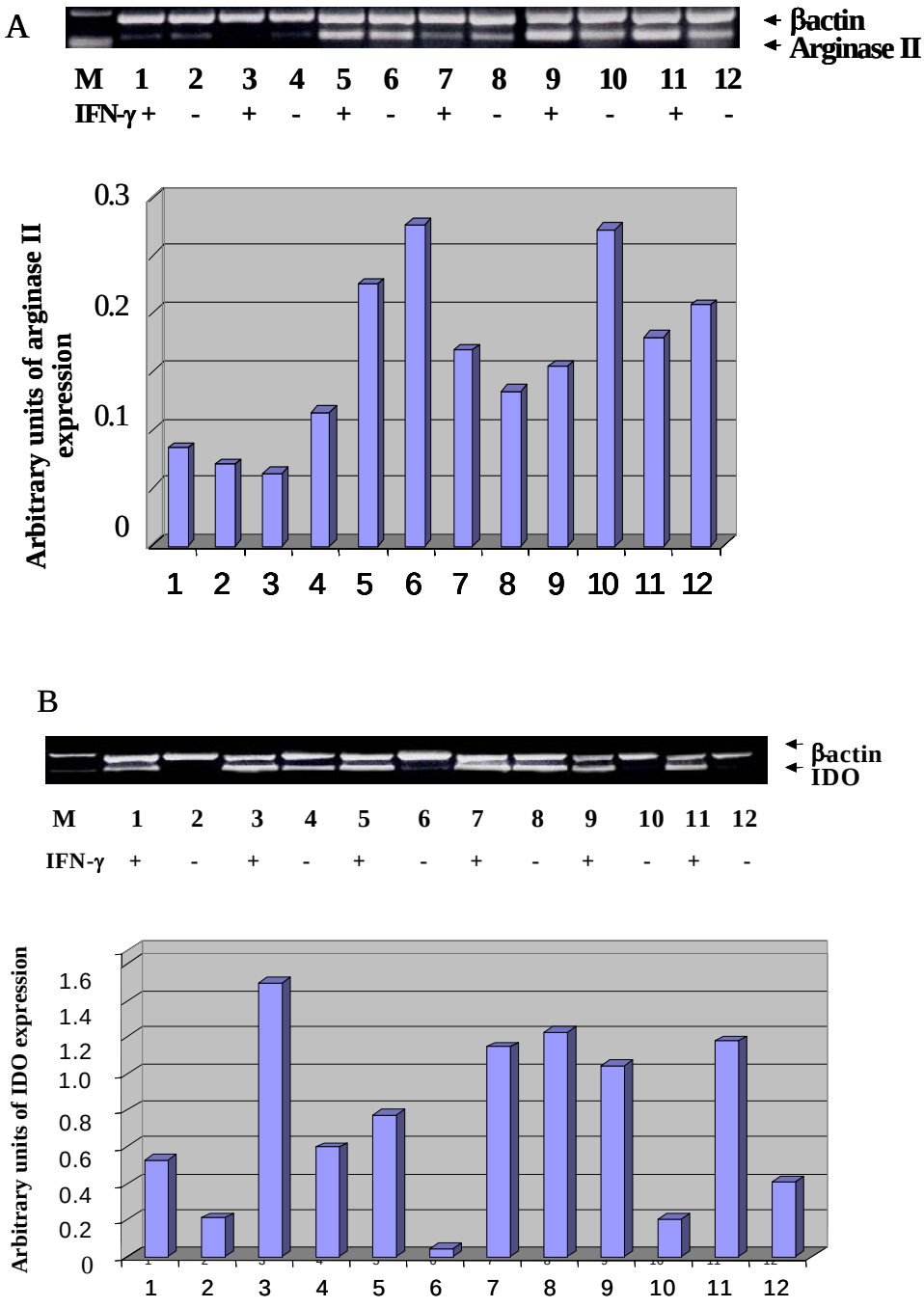


**Figure 2**
**Expression of arginase II (A) and IDO (B) in EOC cell lines.** The cells were treated with rIFN-γ or left untreated. RNA was extracted and RT-PCR performed as described in Materials and Methods. Shown are the results obtained for HEY cells treated with rIFN-γ (lane 1), control untreated HEY cells (lane 2), OVCAR3 cells treated with rIFN-γ (lane 3), control OVCAR3 cells (lane 4), CAOV3 cells treated with rIFN-γ (lane 5), control CAOV3 cells (lane 6), SKOV3 cells treated with IFN-γ (lane 7), control SKOV3 cells (lane 8), 2008.13 treated with rIFN-γ (lane 9), control 2008.C13 cells (lane 10), 2008 cells treated with rIFN-γ (lane 11), control 2008 cells (lane 12). M: molecular weight marker.

**Table 1: Arginase activity in EOC cell lines**

| Cell line | Ki67 (%) | Arginase II RT-PCR | Arginase activity (μg urea/mg protein) – control | Arginase activity (μg urea/mg protein) – rIFN-γ-treated cells | Cytostasis (%) |
|---|---|---|---|---|---|
| 2774* | 95 | + | 1.79 | 0.35 | 47.2 + 11.9 |
| CAOV3 | 93 | + | 1.00 | 0.51 | 80.9 + 6.2 |
| NMP-1 | 84 | + | 0.36 | 0.09 | 83.6 + 6.9 |
| 2008* | 31 | + | 0.69 | 4.90 | 70.8 + 13.8 |
| 2008.C13 | 34 | + | 1.00 | 3.02 | 54.2 + 15.4 |
| HEY | 91 | +/- | 0.14 | 0.14 | 57.8 + 22.6 |
| SKOV3 | 72 | +/- | 0.18 | 0.18 | 7.5 + 4.1 |
| OVCAR3 | 54 | +/- | 0.08 | 0.09 | 44.1 + 19.1 |

The percentage of Ki-67 was determined by immunocytochemistry, and the expression of arginase II was examined by RT-PCR as described in Materials and Methods. Arginase activity and growth inhibition were determined after 72 hours of incubation with control medium or rIFN-γ and are representative of 4 independent experiments. rIFN-γ was added daily to the cells. *Platinum-sensitive.

**Table 2: Effect of $N^G$-methyl-L-arginine on inhibition of arginase activity by rIFN-γ**

| Cell line | Arginase activity (μg urea/mg protein) | | | |
|---|---|---|---|---|
| | Control | NMMA (1 mM) | rIFN-γ (500 U/ml) | rIFN-γ + NMMA |
| 2774 | 1.8 | 1.7 | 1.0 | 0.7 |
| CAOV3 | 2.1 | 1.5 | 0.8 | 0.6 |
| NMP-1 | 0.5 | 0.5 | 0.1 | 0.1 |
| 2008 | 1.2 | 1.3 | 2.1 | 2.4 |
| 2008.C13 | 1.6 | 1.6 | 2.6 | 3.4 |

The cells were incubated with control medium, rIFN-γ, and/or NMMA for 72 hours and processed as described in Materials and Methods.

the EOC cell lines (Figs. 2A and 2B), was increased by more than two-fold in 5 of the 6 ovarian cancer cell lines treated with IFN-γ (Fig. 2B). In contrast to arginase II and IDO, arginase I and iNOS were not detected in any of these cell lines when cultured in medium alone or with rIFN-γ (data not shown).

rIFN-γ produced significant growth inhibition in 7 of 8 cell lines examined, the exception being SKOV3, a cell line with negligible arginase activity (Table 1). Since the growth-inhibitory activity of rIFN-γ in certain EOC lines appeared to be accompanied with arginase inhibition, and since formation of ornithine from arginine catalyzed by arginase represents the first step in the synthesis of polyamines, we hypothesized the inhibition of arginase activity might be at least partly responsible for the growth-inhibitory effects of rIFN-γ on EOC cell lines. However, the addition of polyamines, ornithine, putrescine, or spermidine did not abolish the growth-inhibitory activity of rIFN-γ (Fig. 3). In addition, there was no interference with growth inhibition by rIFN-γ in EOC cell lines after treatment with $N^G$-hydroxy-L-arginine, an arginase inhibitor.

## Conclusions

This study has shown that EOC cell lines have variable levels of arginase activity, which was also observed in cell lines with elevated levels of the Ki-67 proliferation marker. Among the cell lines examined, CAOV3 and 2008 are considered platinum-sensitive, SKOV3 and 2774 are moderately sensitive, and HEY, 2008.C13 and NMP-1 are platinum-resistant. OVCAR3, the parental line of NMP-1 is also considered platinum-resistant. Elevated arginase activity was observed in both platinum-sensitive 2008 cells and the platinum-resistant subline, 2008.C13, and it was substantially higher in NMP-1 cells than in the parental line, OVCAR3, both platinum-resistant. Although arginase activity was higher in 2008.C13 and NMP-1 cells, platinum-sensitive cell lines, e.g., CAOV3, exhibited elevated arginase activity, while some platinum-resistant lines, e.g., HEY, showed reduced arginase activity. It therefore appears that there is no direct association between arginase activity and platinum-resistance. iNOS expression was not detected in any of the 8 cell lines examined, even after treatment with rIFN-γ. In contrast, IDO transcripts were expressed by 5 of the 6 EOC lines treated with

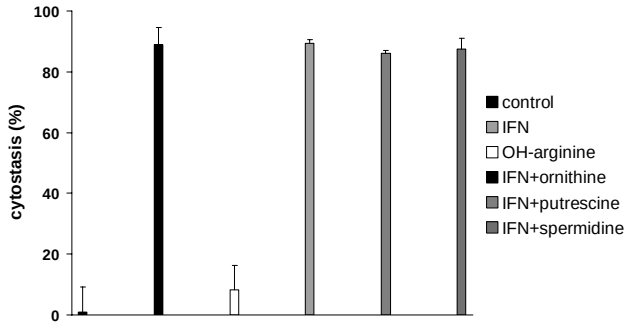


**Figure 3**
**Cytostatic activity of IFN-γ, $N^G$-hydroxy-L-arginine and the effect of polyamines on IFN-γ-induced cytostasis.** NMP-1 cells were seeded at $10^4$ cells/well into 96-well plates in RPMI 1640 with 10% fetal calf serum and cultured overnight. The next day, the cells were washed with phosphate buffered salines, and the medium was replaced with a low-arginine, serum-free medium (5% bovine serum albumin, 1.5% Iscove's modified Dulbecco's medium [GIBCO], 0.2% glucose, 0.01% magnesium sulfate, 0.016% calcium chloride, transferrin 5.5 μg/ml, and insulin 4 μg/ml in Hank's balanced salt medium [GIBCO], pH 7.4) alone or with rIFN-γ (500 U/ml). The arginine concentration of this medium was 6 μM, lower than plasma concentration (normal range, 50–126 μM [38]. After another 24 hours, rIFN-γ (500 U/ml), $N^G$-hydroxy-L-arginine (250 μM), or rIFN-γ plus ornithine (1 mM), putrescine (1 mM), or spermidine (0.5 mM) was added. The cells were then pulsed with [$^3$H] thymidine and incubated for another 24 hours. The cytostatic activity was determined as described in Materials and Methods. Similar results were observed with other EOC cell lines and when experiments were done with RPMI 1640 supplemented with 10% fetal calf serum. Data shown are representative of 3 separate experiments.

rIFN-γ. These results showing no iNOS expression differ from other reported findings in human tumor cell lines, including EOC [28,30–32]. iNOS has been previously detected in some EOC lines but was absent in other cell lines, such as the 2774 cell line [33]. iNOS expression was detected in AD10, a doxorubicin-resistant subline, but not in the parental A2780 EOC line [34]. In another study, iNOS activity was detected after OVCAR3 cells *in vitro* were treated with a combination of rIFN-γ, interleukin-1, and tumor necrosis factor-α [33,35]. In our experiments, rIFN-γ was not combined with other cytokines, which may explain the lack of expression of iNOS.

Treatment of the EOC cell lines with rIFN-γ produced different effects on arginase activity, with inhibition of arginase activity in 3 cell lines, 2774, NMP-1, and CAOV3. In contrast, arginase activity increased after rIFN-γ treatment of 2008 and 2008.C13 cells. rIFN-γ induces iNOS, and $N^G$-hydroxy-L-arginine, an intermediate in NO synthesis by iNOS, is a potent inhibitor of arginase [3,4]. In addition, NO inhibits ornithine decarboxylase in the Caco-2 colon cancer cell line [36]. However, iNOS was not detected by RT-PCR after EOC cells were treated with rIFN-γ, and the addition of NMMA did not abolish the inhibition of arginase activity by rIFN-γ, making it unlikely that the simultaneous induction of iNOS could be responsible for the inhibition of arginase activity in the cell lines. The mechanism(s) of arginase inhibition by rIFN-γ have still to be elucidated. From the present data, regulation of arginase activity by rIFN-γ at the transcriptional level cannot be excluded. There could be different regulatory effects operating in different cell lines, as shown by the results of our experiments. Future experiments could address the mechanism of transcriptional regulation of arginase II by rIFN-γ.

In contrast to the effects of rIFN-γ on arginase II transcript expression, treatment with rIFN-γ increased or induced the expression of IDO. This is in agreement with other reports on the effects of rIFN-γ on various tumor cell lines [16,17], including EOC [13]. Consistent with an increase in the expression of IDO, it has been shown that, at least in some experimental systems, the depletion of L-tryptophan is responsible for the antiproliferative activity of rIFN-γ, and rIFN-γ-mediated growth inhibition could be reversed by adding L-tryptophan [16,17]. The depletion of L-tryptophan is also thought to be responsible for the growth-inhibitory activity of rIFN-γ in certain *in vivo* models [13,14]. However, L-tryptophan depletion is not the only mechanism responsible for the antiproliferative activity of rIFN-γ [17]. Metabolites resulting from L-tryptophan catabolism by IDO (e.g. kynurenine, 3-hydroxykynurenine, or picolinic acid), may also exhibit antitumor activity [29].

In our experiments, the growth-inhibitory activity of rIFN-γ was sometimes, but not always accompanied by the inhibition of arginase activity. Formation of ornithine from L-arginine catalyzed by arginase is the first step in the synthesis of polyamines. The induction of STAT1 and $p21^{WAF1/CIP1}$ mediates growth inhibition by rIFN-γ [15,37,38], and $p21^{WAF1/CIP1}$ is involved in growth inhibition induced by polyamine depletion [8,39,40]. In addition, the inhibition of arginase activity by $N^G$-hydroxy-L-arginine causes growth inhibition in breast cancer cell line MDA-MB-468 [28] and colon adenocarcinoma cell line Caco-2 [36]. We therefore hypothesized that the inhibition of arginase activity might represent another mechanism, at least partly, responsible for the growth-inhibitory effects of rIFN-γ on EOC cell lines.

Experiments in a medium rich in arginine and at arginine concentrations significantly lower than physiological levels [41], however, do not support this hypothesis. Although rIFN-γ treatment results in a decreased transport of polyamines, supraphysiologic concentrations of polyamines used in the current experiments would be expected to compensate for a decrease in the range of 50% that was reported earlier [42]. In addition, no growth-inhibition could be induced by $N^G$-hydroxy-L-arginine. Thus, the decreased production of ornithine induced by rIFN-γ in tumor cells probably does not result in a lowering of polyamine concentrations that would affect the proliferation of the cultured EOC tumor cells. This is in agreement with the experimental data that demonstrate that ornithine in tumor-bearing animals is largely produced in the liver but not in tumor tissues [43]. In addition, rIFN-γ had significant growth inhibitory effects and actually increased, rather than decreased, arginase activity in the 2008 and 2008.C13 cell lines. Arginase I, a cytosolic enzyme, is expressed mostly in the liver, in contrast to arginase II, which is located in the mitochondria [44]. This difference in subcellular localization of the two isoenzymes determines whether ornithine produced by arginase is more likely to be metabolized by ornithine decarboxylase (a cytosolic enzyme) to polyamines or by ornithine aminotransferase (a mitochondrial enzyme) to pyrroline-5-carboxylate and subsequently to proline or glutamate. Production of polyamines appears to depend mostly on arginase I [45–47]. These findings suggest that arginase II expression by tumor cells may not be as relevant to polyamine synthesis and the regulation of tumor cell proliferation. In an earlier study, a marked growth inhibition by $N^G$-hydroxy-L-arginine was observed in a cell line expressing only arginase I, but not in cell lines expressing only arginase II [28].

Regulation of alternative pathways of arginine metabolism has been demonstrated by TH1/TH2 cytokines in monocytes [10,11]. While proinflammatory cytokines (e.g., rIFN-γ) induce iNOS [9], arginase I is induced by TH2 cytokines (e.g., interleukin-4) [48]. Little is known about the effects of cytokines on arginase activity in tumor cells. Inhibition of arginase activity by rIFN-γ could be of importance in the tumor microenvironment as utilization of arginine may be switched to NO synthesis. Arginase has been shown to have immunosuppressive activity *in vitro* [49]. In addition, arginine has been reported to increase NK cell activity both *in vitro* and *in vivo* [50], and a suppression of NK cell activity was observed after arginase treatment [51]. Inhibition of arginase activity by rIFN-γ may thus potentiate other effects of this cytokine on the host immune system. Since rIFN-γ actually increased arginase activity in the 2008 cell line and its cisplatin-resistant subline, 2008.13, it is possible that rIFN-γ induces a paradoxical increase in arginase activity in some tumor systems. This increase in arginase activity in turn might counterbalance the stimulatory activity of this cytokine on the cells of the host innate and adaptive immune systems.

In conclusion, arginase expression and activity varied among EOC cell lines. Arginase activity decreased after rIFN-γ treatment in 3 of the cell lines and increased in 2008 and 2008.C13 cells. The mechanism of arginase inhibition is not dependent on NO synthesis. Arginase II appears to be the isoenzyme responsible for the arginase activity in EOC cells. The growth-inhibitory effects of rIFN-γ treatment on EOC cell lines does not appear to be associated with the inhibition of arginase activity. These findings, however, do not discount the possibility that elevated arginase activity in EOC cells and its modulation by rIFN-γ *in vitro* might be somehow relevant to the regulation of the host-tumor interaction in the tumor microenvironment *in vivo*.

## Authors Contributions

BM designed the experiments, performed the measurements for arginase activity, and drafted the manuscript. WH performed the RT-PCR experiments. RP performed the immunoperoxidase staining. KM performed the growth-inhibition experiments. STG carried and prepared the cell lines. RF acted as Program Director and mentor. All authors read and approved the final manuscript.

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